

# Sequence variations in EBNA-1 may dictate restriction of tissue distribution of Epstein–Barr virus in normal and tumour cells

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In seropositive individuals Epstein–Barr virus (EBV) establishes a virus reservoir in peripheral blood lymphocytes (PBLs). Transmission from one individual to another occurs via saliva due to a lytic (virion productive) phase of infection in the oropharynx. EBNA-1 is responsible for maintaining viral episomes in the host cell and could, therefore, also affect the persistence of the virus in different cell lineages. Based on sequence analysis of EBNA-1 we now demonstrate that (i) in addition to the prototype EBNA-1 (identical to the B95.8 virus EBNA-1), EBV in normal individuals encompasses multiple EBNA-1 subtypes, both in PBLs and in oral

secretions; (ii) although EBV with prototype EBNA-1 is the predominant virus in normal individuals, it is very rarely associated with either nasopharyngeal carcinoma (NPC) or Burkitt's lymphoma (BL); (iii) EBV with an EBNA-1 subtype (V-val) frequently associated with NPC is also selectively detected in oral secretions and not in PBLs; (iv) EBV with the EBNA-1 subtype V-pro is restricted to PBLs, while a mutated version of this subtype is present in BL, but not in NPC. These findings suggest that the variations in EBNA-1 may be relevant to the ability of EBV to persist in different cell types, and hence relevant to its oncogenic potential.

## Introduction

Epstein–Barr virus (EBV) infects about 90% of the world's population (Klein, 1994). In normal seropositive individuals EBV maintains a reservoir in circulating B cells, in which EBV replication is severely restricted. EBV is transmitted predominantly in saliva, and viral particles are readily detected in oral secretions (OS) (Yao *et al.*, 1985). The existence of preferentially replicative and latent virus strains has never been demonstrated and it must be presumed that EBV enters a lytic cycle in lymphocytes within lymphoid tissue in the pharynx, or after infection of pharyngeal epithelial cells (Miller, 1990; Allday & Crawford, 1988). Recently, two groups have been unable to demonstrate the presence of EBV in pharyngeal epithelial cells in normal individuals or patients with infectious mononucleosis by *in situ* hybridization [using Epstein–Barr

virus-encoded RNA (EBER) to detect latently infected cells] (Anagnostopoulos *et al.*, 1995; Tao *et al.*, 1995). However, transient (lytic infection) or a low incidence of infection of normal epithelial cells cannot be ruled out, and EBV is clearly capable of infecting epithelial cells in some circumstances (Knox *et al.*, 1996).

EBV is also associated with a broad range of human malignancies including Burkitt's lymphoma (BL) (Magrath *et al.*, 1993) and nasopharyngeal carcinoma (NPC) (zur Hausen *et al.*, 1970). In recent reports we and others have described several variants of EBNA-1 relative to the prototype virus B95.8 (Bhatia *et al.*, 1996; Wright *et al.*, 1995; Snudden *et al.*, 1995). Each of these EBNA-1 variants has been identified as the exclusive virus subtype in tumour biopsies and/or tumour or lymphoblastoid cell lines known to carry monoclonal EBV, strongly suggesting that these variants represent distinct virus subtypes. We have, therefore, classified EBV into five subtypes, based on the EBNA-1 sequence in the carboxy-terminal region, which we have named according to the amino acid at position

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469                    480                    500                    520  
P-ala  
RGQGSNPKFE NIAEGLR ALLARSHVERTTD EGTWVAGVFVYGGSKTSLYN LRRGTALAIPO  
  
P-thr  
RGQGSNPKFE NIAEGLR TLLARSHVERTTD EGTWVAGVFVYGGSKTSLYN LRRGIALAIPO  
  
V-val  
RGQGSNPKFE NIAEGLR VLLARSHVERTTE EGNWVAGVFVYGGSKTSLYN LRRGIALAVPO  
  
V-pro  
RGEGSNQKFE SIAEGLR PLLARCHVERTTE DGNWVAGVFVYGGSKTSLYN LRRGIGLAIPQ  
  
V-leu  
RGEGSNQKFE SIAEGLR LLLARSHVERTTE DGNWVAGVFVYGGSKTSLYN LRRGIGLAIPQ

Fig. 1. Partial amino acid sequences of the region spanning amino acids 469–530 in the carboxy terminus of EBNA-1 in the various subtypes of EBV. Changes with respect to the prototype B95.8 sequence are shown in bold type.

487 in EBNA-1, since this amino acid residue is highly predictive of the pattern of mutations observed (Fig. 1). We have called two of the subtypes ‘prototypes’. One is P-ala, whose EBNA-1 sequence is identical to that of the B95.8-derived virus, and the second, P-thr, differs slightly from the B95.8 sequence (P-ala) in the carboxy-terminal region. We have referred to the three other subtypes as ‘variants’ (V-pro, V-leu and V-val) because they differ more markedly from P-ala than does P-thr. Snudden *et al.* (1995) identified the EBNA-1 subtype we have referred to as V-val in NPC biopsies, but their report does not indicate whether this subtype is specific to tumour cells in such patients and does not provide information on the frequency of the variant EBNA-1 subtypes in NPC.

To date, EBNA-1 subtypes have not been studied in saliva. In our previous report (Bhatia *et al.*, 1996), we were unable to identify V-val in peripheral blood lymphocytes (PBLs). We have, therefore, examined sequence variations in EBNA-1 in saliva and peripheral blood in 21 individuals to determine whether the V-val subtype can be detected among virus particles released by cells present in the pharynx or salivary glands. In addition, we have examined NPC and BL, as well as PBLs, from various geographical regions.

Methods

■ **Tumour samples.** DNA from NPC tumour tissue was obtained from patients presenting at the Faculty of Medicine, University of Hong Kong and Institut Gustave Roussy, France. DNA from BLs was extracted from frozen biopsies and cell lines from Africa, South America and USA.

■ **Normal samples.** Twenty ml of heparinized peripheral blood was obtained from 53 normal seropositive individuals and PBLs were isolated using Ficoll–Hypaque density gradient centrifugation. OS from 21 of these individuals, obtained by gargling for 2 min with 10 ml phosphate-buffered saline, were also examined. The mouth washes were lyophilized and the residual material used for DNA extraction by cell lysis and digestion with proteinase K according to standard techniques.

The EBNA-1 genotype was determined by PCR and direct sequence analysis of the PCR product as described previously. For at least one of each of the individual subtypes, in addition to the direct sequencing, sequence information was confirmed by plasmid sequencing after cloning the amplified products in a TA vector (Invitrogen).

Results

Pattern of EBNA-1 subtypes in peripheral blood lymphocytes and lymphoblastoid lines from healthy individuals

We have previously reported sequence variations in the carboxy region of EBNA-1 from EBV in PBLs from 32 healthy individuals. The sequence of EBNA-1 in 14 of these 32 individuals was homogeneous (either P-ala or P-thr), whereas 18 individuals demonstrated sequence heterogeneity with multiple bands at the same codon positions in sequencing gels, indicating the presence of a mixture of EBNA-1 subtypes. Based upon the codons in which variations were observed, the presence of specific subtypes in the mixtures could be readily discerned (Bhatia *et al.*, 1996). In the DNA samples obtained from PBLs from an additional 21 healthy individuals, we have now detected the same three EBV subtypes, P-ala, P-thr and V-pro (see Fig. 1 for sequence differences among the subtypes), and again, more than half of the test subjects (13/21) carried EBV with multiple EBNA-1 subtypes (Table 1). The compiled results from the two studies show that the prototype P-ala was the most frequent EBV subtype observed in PBLs, being present in 75 % of normal individuals (40/53). The P-thr and V-pro subtypes were detected in 29 (55 %) and 21 (40 %) normal individuals, respectively. More than half of the test subjects (31/53) were infected with multiple EBV subtypes, the remaining 22 carried either P-ala (10) or P-thr (12). P-ala and P-thr were both detected in 10 subjects. Of considerable interest was the observation that V-pro (readily apparent because of additional amino acid substitutions, for example codons 471, 480 and 500; see Fig. 1) was never detected in isolation, but only in the presence of one of the prototype EBNA-1s, either P-ala (14), P-thr (1) or both (6). V-leu, which we previously detected in BL, and V-val, reported previously by Snudden *et al.* (1995) in NPC, were not detected in any of the samples. There was no apparent difference in the patterns

Table 1. Distribution of EBNA-1 subtypes in matched peripheral blood lymphocytes and oral secretions from 21 test subjects

EBNA-1 subtype (no. of individuals)	
PBL	OS
P-ala (6)	P-ala (5)
	P-ala + V-val (1)
P-thr (2)	P-thr (2)
P-ala + P-thr (8)	P-ala + P-thr (4)
	P-ala + P-thr + V-val (4)
P-ala + V-pro (4)	P-ala (4)
P-ala + P-thr + V-pro (1)	P-ala + P-thr (1)

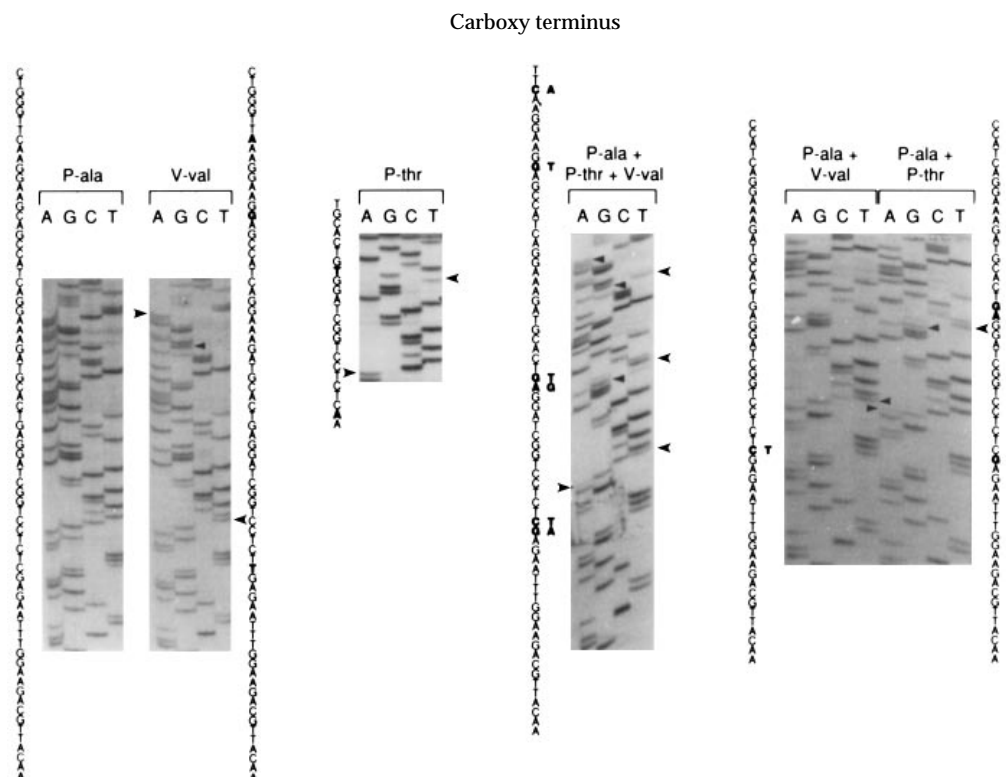


Fig. 2. Examples of sequencing data representing part of the carboxy terminus obtained from sequencing of EBNA-1-amplified DNA from oral samples with various subtypes of EBV. The two panels on the right illustrate direct sequencing reactions of samples with mixtures of (i) P-ala, P-thr and V-val; (ii) P-ala and V-val; and (iii) P-ala and P-thr. The three panels on the left illustrate sequencing data from subcloned PCR products obtained from the amplification of EBNA-1 from oral samples. Base changes with reference to prototype EBNA-1 (P-ala) and the occurrence of multiple bases are shown in bold type and indicated by arrowheads.

of EBNA-1 variants in lymphocytes obtained from individuals in the USA (40), Nigeria (6) or South America (7).

We have also examined 26 spontaneously derived lymphoblastoid cell lines (LCLs) and have not been able to detect either V-pro or V-val in any; P-ala was detected in 10 and P-thr in 16 of these cell lines. In each LCL only a single subtype was present.

#### EBV with the V-val EBNA-1 subtype is detected in oral secretions from healthy individuals

Since the major route of transmission of EBV is via OS, we sequenced the EBNA-1 carboxy region of EBV from the saliva of 21 of the individuals whose PBLs we had examined. As with PBLs, nucleotide sequences were consistent with the presence of one or more EBNA-1 subtypes in each sample. There was general concordance between EBV subtypes present in PBLs and those detected in the oropharyngeal secretions (Table 1) with two significant exceptions: (i) individuals who carried the V-pro subtype in their PBLs did not show the presence of this subtype in OS and (ii) the V-val subtype was detected in the throat washings of five (24%) of these subjects whose lymphocytes contained either P-ala alone or P-ala and P-thr (Fig. 2 and Table 1). In none of these five subjects was V-val

detectable in simultaneously obtained PBLs. All OS samples in which V-val was detected were obtained from subjects in the USA.

#### V-val EBNA-1 from normal oral secretions carries substitutions in the amino-terminal region identical to those in the V-val identified in NPC biopsies

Analysis of the amino terminus of EBNA-1 by Snudden *et al.* (1995) indicated that the EBNA-1 subtypes identified in NPCs also contained changes at amino acids 16 (Glu-Gln), 18 (Gly-Glu), 20 (Thr-Ser), 24 (Glu-Asp) and 27 (Gly-Ser) compared to P-ala EBNA-1. It was apparent from their data that the substitutions at amino acids 16 and 18 were common to both the EBNA-1 subtypes we designated V-val and P-thr, but the change at amino acid 20 (Thr-Ser) was unique to the V-val subtype and the changes at amino acids 24 (Glu-Asp) and 27 (Gly-Ser) were only present in the single P-thr subtype they described.

In the absence of normal cell lines or virus isolates from normal cells that contain the V-val EBNA-1, analysis of concordance between the amino-terminal and the carboxy-terminal substitutions for this subtype can only be performed

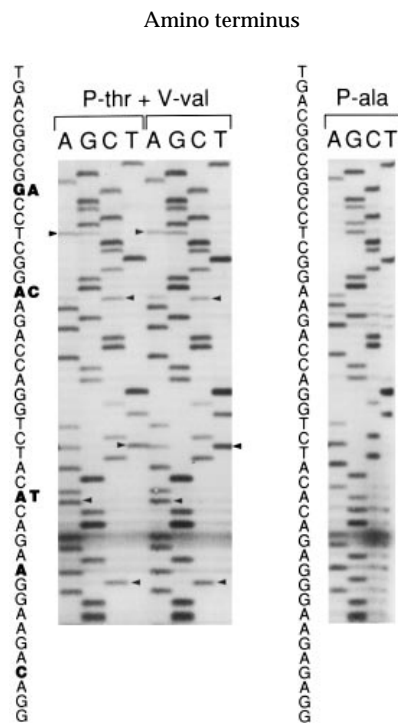


Fig. 3. Sequence data from the amino-terminal region of EBNA-1 in OS samples. Amino-terminal region of EBNA-1 was amplified from DNA obtained from OS and the amplified products were directly sequenced. Panel on the left is a sequence from two of the three OS samples that contained a mixture of substitutions in the amino terminus typical of P-thr and V-val (see Fig. 4). For comparison, the amino-terminal sequence of EBNA-1 in PBLs with the P-ala subtype is given in the right-hand panel. Substitutions and heterogeneity are shown in bold type and indicated with arrowheads.

in OS samples that contain mixed EBNA-1 subtypes. Furthermore, the presence of an Ala-Gly repeat region between the amino terminus and the carboxy terminus prevents efficient amplification of the complete region as a single DNA fragment.

We therefore sequenced a DNA fragment from nucleotide –9 (relative to A of ATG) to nucleotide 270, spanning the entire amino-terminal region of EBNA-1 in saliva from healthy individuals. Examination of these data demonstrated substitutions at amino acids 16, 18, 20, 24 and 27 in several OS samples (Figs 3 and 4). However, the variation at amino acid 20 (Thr-Ser), which was characteristic of the V-val subtype seen in NPCs, was observed in only four of the five OS samples which, in addition to other substitutions, also contained valine at position 487.

Substitutions at codons 24 and 27 occur in samples that carry a P-thr subtype EBNA-1

We wished to determine whether the substitutions at codons 24 and 27 reported by Snudden *et al.* (1995) in the EBNA-1 from the C-15 cell line derived from NPC were always concordant with the presence of P-thr in the carboxy terminus. We hence sequenced the amino terminus of EBNA-

Sample (number)		Amino acids		
		487-500	16-28	
PBL				
P-ala	(3)	ALLARSHVERTTDE	EKGDTSGPEGSGG	
P-thr	(1)	TLLARSHVERTTDE	QKEDTSGPDGSSG	
P-ala+ P-thr	(1)	A TLLARSHVERTTDE	Q E D S EKGDTSGPEGSGG	
P-thr+V-pro	(1)	P C ED TLLARSHVERTTDE	EKGDTSGPEGSGG	
P-ala+P-thr+V-pro	(2)	T C ED ALLARSHVERTTDE P	QKEDTSGPDGSSG	
OS				
P-ala+P-thr +V-val	(3)	T E ALLARSHVERTTDE V	S D S QKEDTSGPEGSGG	
P-ala+V-val	(1)	V E ALLARSHVERTTDE	S D S QKEDTSGPEGSGG	
P-ala+P-thr	(5)	T ALLARSHVERTTDE	EKGDTSGPEGSGG	
LCL				
P-thr	(13)	TLLARSHVERTTDE	QKEDTSGPDGSSG	
Consensus sequence for the amino terminal region (amino acids 1-36) of various subtypes				
	1	10	20	30
P-ala	MSDEGPGTG	PGNGLGEKGD	TSGPEGSGGSG	PQRRGG
P-thr	MSDEGPGTG	PGNGLGQKED	TSGPDGSSGSG	PQRRGG
V-val	MSDEGPGTG	PGNGLGQKED	SSGPEGSGGSG	PQRRGG

Fig. 4. Correlation of amino acid substitutions in the amino-terminal region of EBNA-1 (residues 16–28) with substitutions in the carboxy region that define the EBNA-1 subtype (P-ala, P-thr, V-pro and V-val) in EBV genomes present in normal PBLs, OS samples or in spontaneously derived LCLs. The amino acid substitutions are derived from DNA-based sequence analysis. Where DNA sequence analysis indicated the presence of heterozygous nucleotides, alternative amino acids are shown at the same position.

1 from eight PBLs (five of which contained P-thr) and nine OS (eight of which contained P-thr) samples (Fig. 4). Of the eight (four PBLs and four OS) samples that carried the characteristic substitutions at amino acids 24 and 27, the carboxy region of EBNA-1 in seven was consistent with the P-thr subtype. Another six samples that contained P-thr (five OS and one PBLs) did not carry the substitutions at codons 24 and 27. We next sequenced the amino-terminal region in 13 spontaneous LCLs that were established from PBLs of two individuals in which we had determined the carboxy region of EBNA-1 to be identical to the P-thr subtype; we found that the substitutions at codons 24 and 27 were present in all 13 spontaneous LCLs (Fig. 4). Thus, the substitutions at amino acids 24 and 27 occurred preferentially in association with the P-thr subtype EBV.

PCR products obtained by amplification of the amino terminus from two independent OS samples (V-val and P-thr) and two LCLs that contained P-thr were cloned in a TA vector and the inserts from subcloned plasmids were independently

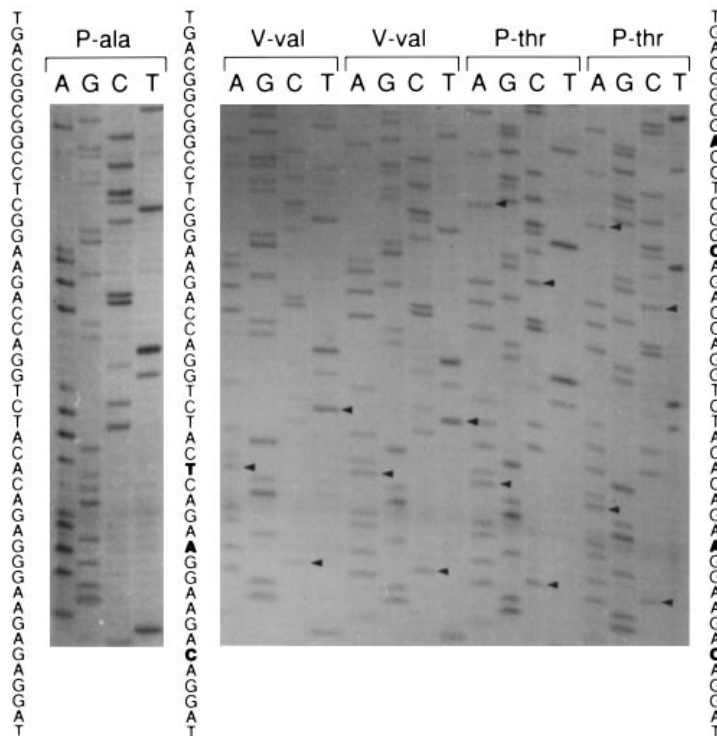


Fig. 5. The panel on the right depicts amino-terminal EBNA-1 fragments from OS samples shown in Fig. 3 that were subcloned following PCR amplification and independently sequenced. The panel on the left shows sequencing data obtained from the subcloned P-ala fragment shown in Fig. 3. Substitutions are shown in bold type and indicated by arrowheads.

sequenced. These data, shown in Fig. 5, demonstrated that the subclones contained only the substitutions at amino acids 16, 18 and 20 (i.e. as found in V-val from NPC), or the substitutions at amino acids 16, 18, 24 and 27 (i.e. as in P-thr from BL or NPC) or contained no substitutions. None of the subclones contained a subset or an admixture of the prototype and the two sets of substitutions.

#### Prototype EBNA-1 is rarely detected in EBV-associated tumours

Table 2 is a summary of carboxy- and amino-terminal EBNA-1 sequences from BL and NPC tumours. In every case, only a single subtype was observed in each tumour (i.e. in no case were multiple bands present at the same position), whether BL or NPC. V-leu was present only in the BL samples and V-val only in the NPCs. All 14 BL samples that were typed as P-thr with respect to the carboxy terminus also contained the concordant substitutions at the amino terminus. Only one of the two P-ala-containing tumours was truly prototype in both the amino and carboxy regions. The other contained an amino terminus consistent with P-thr. Of 11 V-leu samples, eight contained amino-terminal sequences typical of P-ala and three contained the substitutions at amino acids 24 and 27, typical of P-thr. None of the BL samples contained amino-terminal substitutions found in NPCs or OS containing V-val. Of 13 NPC samples that were analysed for EBNA-1 sequences in both the carboxy and amino termini, nine samples were typically V-val in both the regions, three samples were identical to P-thr and one sample contained a carboxy region

Table 2. EBNA-1 carboxy- and amino-terminal variations in tumours

Tumour samples	Carboxy region	Amino region
<b>Burkitt's lymphoma</b>		
1	P-ala	P-ala
1	P-ala	P-thr
14	P-thr	P-thr
1	V-pro	P-ala
8	V-leu	P-ala
3	V-leu	P-thr
<b>Nasopharyngeal carcinoma</b>		
9	V-val	V-val
3	P-thr	P-thr
1	P-thr	P-ala

identical to P-thr and amino-terminal sequences identical to P-ala. None of the NPC samples contained either V-leu or V-pro. Thus, only one (a BL from North Africa) of the 41 tumour samples examined contained a true prototype (P-ala) EBNA-1.

#### Discussion

Traditionally, EBV has been divided into type I and type II strains. These strains are based upon sequence variations in EBNA-2 and EBNA-3A, 3B and 3C (Addinger *et al.*, 1985; Sculley *et al.*, 1989; Rowe *et al.*, 1989; Sample *et al.*, 1990).

Most isolates of EBV are type I, but type II virus was reported, in a study from Memphis, USA, to be frequently found in OS (Sixbey *et al.*, 1989). Type II EBV has been shown to be present in 40–50% of EBV-associated endemic BL and AIDS-associated lymphomas and lymphomas in the immune-compromised host (Goldschmidts *et al.*, 1992; Boyle *et al.*, 1991). Recently, we and others have described variations in the EBNA-1 sequence (Fig. 1) in EBV isolates from tumours and spontaneously derived LCLs (Bhatia *et al.*, 1996; Snudden *et al.*, 1995; Wraitham *et al.*, 1995). These variations occur with equal frequency in type I and type II strains (Bhatia *et al.*, 1996; Wraitham *et al.*, 1995). The detection of each of the EBNA-1 subtypes as the sole species of EBNA-1 in either tumour samples or immortalized cell lines indicates that each subtype exists as a separate subspecies. Thus, the simultaneous presence in some PBLs and OS samples of clusters of substitutions typical of more than one EBNA-1 subtype strongly suggests that multiple virus subtypes are present in the blood and saliva of a high fraction of normal individuals. This interpretation is further supported by the patterns of substitutions observed in the subcloned inserts from the amplified PCR products (Figs 2 and 4). Each clone contained an EBNA-1 sequence pattern identical to that observed in tumours or cell lines containing a single EBNA-1 subtype. Finally, there is concordance in the substitutions in the amino and the carboxy regions in that the amino region patterns typical of one or the other subtype (e.g. P-thr, V-val) were usually found only in the presence of the expected carboxy region pattern. Substitutions at amino acid 20 appear to be specific for the V-val subtype, and were observed only in OS samples and in NPCs from Hong Kong, whereas substitutions at codons 24 and 27 occurred for the most part in the presence of a threonine at position 487. An exception to this was observed in BL, where a V-leu carboxy terminus was associated either with a B95.8-like amino terminus or the changes in codons 24 and 27 associated in normal cells and NPCs with threonine at codon 487.

Our data are entirely consistent with the possibility that the distinctive set of substitutions observed by us and Snudden *et al.* (1995) in NPC from Hong Kong, which we have referred to as the V-val subtype of EBNA-1, is derived from a virus subtype that is detectable in OS of normal individuals but does not appear to be present in normal lymphocytes. This complements our previous observation (Bhatia *et al.*, 1996; extended in the present report) that BL is frequently associated with an EBNA-1 subtype not found either in normal lymphocytes or OS, although a subset of NPCs and BLs from North Africa carry the P-thr subtype found in both PBLs and throat washings.

There are two possibilities that could account for our finding that multiple viral subtypes (based upon EBNA-1 sequences) are present in the same individual. Either the subtypes arise from a single subtype (that which originally infected the individual) by an ordered and incremental process of substitutions, a phenomenon that has been documented in

the generation of other mutant viruses (Molla *et al.*, 1996) or the subtypes exist as naturally occurring virus subtypes. The latter possibility would require the additional corollary that individuals may be infected by more than one subtype and, moreover, that certain subtypes (V-pro and V-val) can only infect individuals already infected with P-ala or P-thr. This seems inherently unlikely since (i) Ebnotyping is consistent with infection and persistence of a single virus type and (ii) multiple subtypes would presumably have to infect the same cell if some are required to 'help'. This is not only contrary to the current paradigms but would presumably be mediated against by virtue of the very small number of EBV-containing cells in normal individuals.

While at first sight the generation of multiple subtypes from the original infecting virus also seems improbable, since both V-pro and V-val differ consistently at multiple residues from the P subtypes (Bhatia *et al.*, 1996), we favour this possibility as it has ample precedence in the well-described quasi-species of RNA viruses (Duarte *et al.*, 1994; Eigen, 1993). Moreover our failure to detect, to date, V-pro in the LCLs derived spontaneously from PBLs, and the similar absence of this subtype in the cell lines examined by Wraitham *et al.* (1995) suggest that V-pro is not capable of transforming B cells and is therefore unlikely to be transmitted from one individual to another. However, known transformation-defective variants of EBV (for example P3HR1) are not V-pro. The apparent absence of V-pro from saliva is entirely consistent with this possibility, and further suggests that V-pro is also unable to enter into a lytic cycle, although we cannot exclude the possibility that V-pro is present in OS, but in numbers too small to be detected.

The presence of V-val in saliva but not in PBLs also requires an explanation. If epithelial cells do not normally contain EBV, virus must presumably enter saliva from PBLs which enter a lytic phase while passing through pharyngeal lymphoid tissue. The absence of V-val in PBLs makes this an unlikely scenario with respect to this subtype and the possibility that V-val is a subtype particularly adapted to epithelial cells must be considered. This is not inconsistent with the recent failure to identify EBV in epithelial cells since (i) one of these studies was conducted in infectious mononucleosis (IM) (if V-val is derived from P-ala or P-thr *in vivo* it would be unlikely to be present in primary infection) and (ii) if V-val were predominantly lytic in epithelial cells it may prove to be difficult to detect. Alternatively, it is possible that epithelial cells are only rarely infected by V-val, or such cells fail to express EBERs. Either explanation would account for the failure to detect EBV in epithelial cells. One possible explanation for our observation that V-val was detected only in association with P-ala or P-thr or both is that the establishment of a reservoir in lymphocytes is essential for the persistence of EBV in the host. Even if small numbers of epithelial cells were infected by V-val transmitted in saliva from another individual, lysis of such cells would lead to rapid elimination of the infection. Thus, the presence of

significant numbers of V-val particles in OS probably requires continuous generation from P-ala or P-thr. The ability to detect V-val may be indicative of a higher level of lytic infection in the individual, but to examine this question, quantitative assessment of virus in OS would be required. The presence of V-val in saliva is quite consistent, of course, with its association with NPC (Table 2; Snudden *et al.*, 1995), and studies of the frequency of V-val in OS in regions in which this disease is prevalent and its association with other factors believed to predispose to this disease (e.g. consumption of salted fish during infancy) would be worthwhile. Further studies of EBNA-I subtypes in NPC from different world regions is also warranted.

Although the prototype virus (P-ala) is the most prevalent subtype and can be readily detected both in OS and in PBLs from the majority of healthy individuals, it is rarely present in EBV-positive BLs or NPCs. In addition to our data this finding is further supported by the report of Snudden *et al.* (1995) and Wraitham *et al.* (1995) who also described variations of EBNA-I in tumour samples. Wraitham *et al.* (1995) examined five tumour cell lines from BLs, and P-ala was not present in any of these samples. Snudden *et al.* (1995) described the presence of V-val in biopsies from Chinese NPC and P-thr in one NPC xenograft obtained from a North African patient, but again did not observe P-ala in the material. It is possible that this subtype cannot readily infect non-lymphoid cells; however, this does not explain its absence in BLs. Our study of EBNA-I subtypes in BLs (USA, South America, Africa), normal PBLs and OS (USA, South America, Africa) and NPCs (Hong Kong and Africa) indicates that our findings cannot be simply explained on the basis of the prevalence of the various EBNA-I subtypes in different geographic regions. P-ala EBNA-I in normal samples appears to be independent of geography, and is absent in tumour samples from regions in which it is present in the PBLs of 75% of normal individuals. Moreover in a limited series of seven patients with either a P-thr or V-leu EBNA-I subtype in BL tissue, P-ala was readily detected in spontaneous LCLs derived from the patients' normal lymphocytes (data not shown).

In conclusion, our data demonstrate the frequent co-existence of multiple EBV subtypes, based on EBNA-I sequences, in normal immunocompetent individuals and demonstrate tropism of the V-pro and the V-val strains to circulating lymphocytes (latent) and the oropharyngeal (lytic) compartments respectively. The data also highlight the absence of the prototype virus, P-ala, in both BL and NPC. One subtype of EBNA-I, V-leu, which we have so far detected only in BL, appears to be derived from V-pro by a single mutation (Bhatia *et al.*, 1996), but V-val, the subtype that we and Snudden *et al.* (1995) have detected in NPC from Hong Kong, is identical to an EBNA-I subtype present in the saliva of normal individuals from the USA. The observation that North African BL and NPC are associated with the P-thr subtype of EBNA-I is intriguing, particularly since this subtype is

prevalent in PBLs in all regions we have examined to date. The possibility that significant biological differences exist in the tumours from North Africa must be considered. In the case of BL, some support for this notion is provided by our previous findings regarding regional differences in the pattern of chromosomal breakpoint locations. An alternative explanation is that regionally variable environmental or genetic factors influence the oncogenicity of EBNA-I subtypes.

Our findings suggest that either EBNA-I influences the virus life cycle, including cellular tropism, more than has been previously conceived, or that EBNA-I subtypes are associated with genetic polymorphism in other genes that are responsible for the differences we have observed. In either event, and coupled to the predisposition of EBNA-I transgenic mice to lymphoma development (Wilson *et al.*, 1996), our data provide strong support for a direct role of EBNA-I in human cancers.

## References

- Aldinger, H. K., Delius, H., Freese, U. K., Clarke, J. & Bornkamm, G. W. (1985). A putative transforming gene of Jijoye virus differs from that of Epstein-Barr virus prototypes. *Virology* **141**, 121-134.
- Allday, M. J. & Crawford, D. H. (1988). Role of epithelium in EBV persistence and pathogenesis of B-cell tumors. *Lancet* **i**, 855-857.
- Anagnostopoulos, I., Hummel, M., Kreschel, C. & Stein, H. (1995). Morphology, immunophenotype, and distribution of latently and/or productively Epstein-Barr virus-infected cells in acute infectious mononucleosis: implications for the interindividual infection route of Epstein-Barr virus. *Blood* **85**, 744-750.
- Bhatia, K., Raj, A., Gutiérrez, M. I., Judde, J.-G., Spangler, G., Venkatesh, H. & Magrath, I. T. (1996). Variation in the sequence of Epstein-Barr virus nuclear antigen 1 in normal peripheral blood lymphocytes and in Burkitt's lymphomas. *Oncogene* **13**, 177-181.
- Boyle, M. J., Sewell, W. A., Sculley, T. B., Apolloni, A., Turner, J. J., Swanson, C. E., Penny, R. & Cooper, D. A. (1991). Subtypes of Epstein-Barr virus in human immunodeficiency virus-associated non-Hodgkin lymphoma. *Blood* **78**, 3004-3011.
- Duarte, E. A., Novella, I. S., Weaver, S. C., Domingo, E., Wain-Hobson, S., Clarke, D. K., Moya, A., Elena, S. F., de la Torre, J. C. & Holland, J. J. (1994). RNA virus quasispecies: significance for viral disease and epidemiology. *Infectious Agents and Disease* **3**, 201-214.
- Eigen, M. (1993). The origin of genetic information; viruses as models. *Gene* **135**, 37-47.
- Goldschmidts, W. L., Bhatia, K., Johnson, J. F., Akar, N., Gutiérrez, M. I., Shibata, D., Carolan, M., Levine, A. & Magrath, I. (1992). Epstein-Barr virus genotypes in AIDS-associated lymphomas are similar to those in endemic Burkitt's lymphomas. *Leukemia* **6**, 875-878.
- Klein, G. (1994). The paradoxical coexistence of EBV and the human species. *Epstein-Barr Virus Report* **1**, 5-9.
- Knox, P. G., Li, Q. X., Rickinson, A. B. & Young, L. S. (1996). In vitro production of stable Epstein-Barr virus-positive epithelial cell clones which resemble the virus: cell interaction observed in nasopharyngeal carcinoma. *Virology* **215**, 40-50.
- Magrath, I., Jain, V. & Bhatia, K. (1993). Molecular epidemiology of Burkitt's lymphoma. *Epstein-Barr Virus Associated Diseases* **225**, 377-396.
- Miller, G. (1990). Epstein-Barr virus: biology, pathogenesis and medical aspects. In *Fields Virology*, 2nd edn, pp. 1921-1958. Edited by B. N. Fields & D. M. Knipe. New York: Raven Press.

- Molla, A., Korneyeva, M., Gao, Q., Vasasanonda, S., Schipper, P., Mo, H. M., Markowitz, M., Chernyavskiy, T., Niu, P., Lyons, N. & Hssu, A. (1996). Ordered accumulation of mutations in HIV protease confers resistance to ritonavir. *Nature Medicine* **2**, 760–766.
- Rowe, M., Young, L. S., Cadwallader, K., Petti, L., Kieff, E. & Rickinson, A. B. (1989). Distinction between Epstein–Barr virus type A (EBNA 2A) and type B (EBNA 2B) isolates extends to the EBNA 3 family of nuclear proteins. *Journal of Virology* **63**, 1031–1039.
- Sample, J., Young, L. S., Martin, B., Chatman, T., Kieff, E. & Rickinson, A. B. (1990). Epstein–Barr virus types 1 and 2 differ in their EBNA 3A, 3B and 3C genes. *Journal of Virology* **64**, 4084–4092.
- Sculley, T. B., Apolloni, A., Stumm, R., Moss, D. J., Mueller-Lantczh, N., Misko, I. S. & Cooper, D. A. (1989). Expression of Epstein–Barr virus nuclear antigens 3, 4 and 6 are altered in cell lines containing B type virus. *Virology* **171**, 401–408.
- Sixbey, J. W., Shirley, P., Chesney, P. J., Buntin, D. M. & Resnick, L. (1989). Detection of a second widespread strain of Epstein–Barr virus. *Lancet* **2**, 761–765.
- Snudden, D. K., Smith, P. R., Lai, D., Ng, M.-H. & Griffin, B. E. (1995). Alterations in the structure of the EBV nuclear antigen, EBNA1, in epithelial cell tumors. *Oncogene* **10**, 1545–1552.
- Tao, Q., Srivastava, G., Chan, A. C., Chung, L. P., Loke, S. L. & Ho, F. C. (1995). Evidence for lytic infection by Epstein–Barr virus in mucosal lymphocytes instead of nasopharyngeal epithelial cells in normal individuals. *Journal of Medical Virology* **45**, 71–77.
- Wilson, J. B., Bell, J. L. & Levine, A. J. (1996). Expression of Epstein–Barr virus nuclear antigen-1 induces B cell neoplasia in transgenic mice. *EMBO Journal* **15**, 3117–3126.
- Wright, M. N., Stewart, J. P., Janjua, N. J., Pepper, S. D., Sample, C., Rooney, C. M. & Arrand, J. R. (1995). Antigenic and sequence variation in the C terminal unique domain of the Epstein–Barr virus nuclear antigen EBNA-1. *Virology* **208**, 521–530.
- Yao, Q. Y., Rickinson, A. B. & Epstein, M. A. (1985). A re-examination of the Epstein–Barr virus carrier state in healthy seropositive individuals. *International Journal of Cancer* **35**, 35–42.
- zur Hausen, H., Schulte-Holthausen, H., Klein, G., Henle, W., Henle, G., Clifford, P. & Santesson, L. (1970). EBV DNA in biopsies of Burkitt's tumors and anaplastic carcinomas of the nasopharynx. *Nature* **228**, 1056–1058.

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